Plant Physiol. (1977) 59, 658-663

Effect of a *myo*-Inositol Antagonist, 2-O,C-Methylene-*myo*-Inositol, on the Metabolism of *myo*-Inositol-2-^{3}H and D-Glucose-1-^{14}C in *Lilium longiflorum* Pollen[1]

Received for publication Ausust 17, 1976 and in revised form November 15, 1976

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ABSTRACT

2-O,C-Methylene-*myo*-inositol (MMO), a *myo*-inositol (MI) antagonist, inhibits germination and tube elongation of pollen from *Lilium longiflorum* cv. Ace or 44. The presence of 5 mM MMO in Dickinson's pentaerythritol medium (Plant Physiol. 43:1-8) partially blocks germination. The tubes produced are short and fail to elongate. In the presence of MI, MMO's toxic effect is blocked. As little as 0.56 mM MI will maintain normal germination in the presence of 43 mM MMO, and pollen tubes continue to elongate for 2 to 3 hr. Eventually, the toxic action of MMO prevents further growth. MMO does not inhibit UDP-D-glucose dehydrogenase from lily pollen.

Uptake of MI-2-^{3}H and incorporation of tritium into galacturonic acid and pentose units of tube wall pectin are blocked by MMO. The site of this inhibition is undertermined. Uptake of D-glucose-1-^{14}C and incorporation of ^{14}C into 70% ethyl alcohol-insoluble polysaccharides of germinated pollen are not blocked by MMO, but distribution of label into polysaccharide product is altered. In MMO-treated pollen, very little ^{14}C is found in uronic acid or pentose units. At 30 mM MMO, about two-thirds of the carbon flow from D-glucose to these pectic components is interrupted. MMO also alters D-glucose metabolism in the 70% ethyl alcohol-soluble fraction, but the compound involved must still be identified.

These results offer fresh evidence of an intermediary role for MI during UDP-D-glucuronate biosynthesis in germinated pollen.

Conversion of D-glucose 6-P to UDP-D-glucuronate and to products of glucuronate metabolism in higher plants follows either the sugar nucleotide oxidation pathway or the MI[4] oxidation pathway. Singly or together these two processes control production of all hexuronic acid and pentose components required for the biosynthesis of cell walls and related structures. The relative contribution of each to UDP-D-glucuronic acid formation is dependent on the stage of growth and pattern of development of a given plant.

Indications of a functional role for the MI oxidation pathway may be inferred from observations on phytate breakdown during seed germination and seedling development in wheat (12, 13), from cyclic formation and disappearance of L-quebrachitol in sycamore maple (*Acer pseudoplatanus*) as this tree approaches and passes through its dormant period (25), and from periodic changes in MI in *Eucalyptus regnans* due to biosynthetic demands of developing tissue (29, 30). Conversion of D-glucose and MI to pectin and hemicellulose has been examined in some detail using detached root tips from corn seedlings (8, 20, 21), and an attempt has been made to measure the effect of exogenous MI on the conversion of labeled D-glucose to products of UDP-D-glucuronate metabolism (22). In the latter study, less label reached cell wall galacturonosyl units when a high level of MI was supplied to detached 3-day-old corn root tips, suggesting that UDP-D-glucuronate was formed by way of MI. Failure to find UDP-D-glucose dehydrogenase activity (19) favored this view, but a more recent study by Darrow and Knotts (2) reported the presence of this enzyme in corn seedlings. In view of this, the effect of exogenous MI is best interpreted as due to inhibition of UDP-D-glucose dehydrogenase by products of MI-produced glucuronate metabolism (7).

Germinating pollen is a relatively simple biological system to handle and it readily converts MI to tube wall pectin (1, 10, 28). In an unpublished experiment on MI-2-^{3}H metabolism in germinating pear pollen, Stanley and Loewus noted that MMO blocked incorporation of label into pectin. MMO is a MI analog, one of the most effective compounds prepared and tested by Schopfer and Posternak (26) for MI antagonist activity in *Schizosaccharomyes pombe* (4, 5, 15, 17, 18, 27). Weinhold *et al.* (32) found this compound to be toxic when administered intraperitoneally to mice (LD_{50}, 150 mg/kg) or rats (LD_{50}, 100 mg/kg) (32), and the cause of death was attributed to necrotic lesions in kidney cortex. In subsequent studies (31), Weinhold and Anderson showed that MMO inactivated MI oxygenase (EC 1.13.99.1) and blocked the catabolism of MI. Administration of MI along with MMO prevented enzyme inactivation. Hauser *et al.* (9) studied the effects of MMO on D-glucose-U-^{14}C metabolism in brain and kidney slices from young rats. Tissues from MMO-injected animals revealed a decline in MI-synthesizing capacity.

More recently, Deshusses and his colleagues (4-6, 14, 15) examined the biochemical properties and fine structural features of *S. pombe* when this fission yeast was grown in the presence of MMO. They found a large increase in monophosphoinositides and a change in the monosaccharide composition of cell wall polysaccharides but no distinct effect on cellular respiration. MMO interrupted septa formation and induced unilateral elongation of cells. Nuclear division was also inhibited.

In the only study published thus far on exposure of higher plant tissue to MMO, Rubery and Northcote (24) briefly exam-

[1] Scientific Paper No. 4688, Project 0266, College of Agriculture Research Center, Washington State University, Pullman, Wash. 99164. This work was supported in part by Grants GM-12422 and GM-22427 from the National Institute of General Medical Sciences, National Institutes of Health.

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[4] Abbreviations: MI: *myo*-inositol; MMO: 2-O,C-methylene-*myo*-inositol (31) (also referred to as 2,7-epoxy-*myo*-inositol [18], the oxide of 2-C-methylene-*myo*-inositol [4,5,15] and 2:7 anhydro-2-hydroxymethyl-*myo*-inositol [17].

ined the effect of this MI antagonist on the metabolism of D-glucose-^{14}C in starch-depleted, 2,4-D-dependent, cell cultures of *A. pseudoplatanus* (sycamore maple). In the absence of 2,4-D, incorporation of label into arabinosyl units of cell wall polysaccharides of MMO-treated cells was reduced by 50%. Incorporation into galactosyl and xylosyl units also was reduced. When 2,4-D was present, only xylosyl incorporation was affected.

The present paper examines the effects of MMO on uptake and incorporation of label from MI-2-^{3}H and D-glucose-1-^{14}C into germinated pollen of *Lilium longiflorum*. A preliminary report of this research appeared in 1973 (11).

MATERIALS AND METHODS

Pollen from *L. longiflorum* cv. Ace or 44 was collected and stored as described in the preceding paper (1). MI-2-^{3}H, 4.9 Ci/mol, was prepared chemically by reduction of *myo*-inosose-2 with sodium borohydride-^{3}H and recrystalized from water with ethyl alcohol before use. MMO was prepared by the method of Posternak (16). An unknown impurity, judged to be <5% of the main product by GLC, was found in the recrystallized MMO. The hydrolysis product of MMO, 2-hydroxymethyl-MI, was undetectable by GLC.

UDP-D-glucose dehydrogenase (UDP-glucose:NAD oxido reductase, EC 1.1.1.32) was isolated by the procedure of Davies and Dickinson (3) from stored *L. longiflorum* cv. Ace pollen with 50% viable grains. The enzyme had a specific activity of 0.48 U/mg of protein with U defined as 1 μmol of NAD^+ reduced/mg protein·min at 24 C. In the absence of substrate, the presence of 32 mM MMO promoted reduction of NAD^+ equivalent to 0.004 U/mg protein. Assays were made spectrophotometrically at 340 nm in 2-ml samples. The reaction remained linear at least 12 min. Readings were taken at 2-min intervals and initial velocities determined from the slope using appropriate controls for enzyme and MMO.

Five-mg batches of pollen were germinated in 1-ml volumes of Dickinson's pentaerythritol medium at 27 C in 10-ml borosilicate flasks. Pollen grains were held in suspension by gentle agitation on a reciprocal shaker at 30 oscillations/min.

RESULTS

MMO repressed pollen germination and interrupted pollen tube elongation. Media containing 5 mM MMO reduced germination and elongation to 66% of the control during a 5-hr incubation. Those pollen grains that germinated in the presence of MMO failed to elongate when the MMO concentration was greater than 5 mM. To study the effect of MMO on tube elongation, it was necessary to germinate pollen in MMO-free media. Data in Table I were taken from an experiment in which pollen was grown for 5 hr in Dickinson's pentaerythritol media followed by 3 hr in the same media with MMO added. The percentage of germination and tube length were measured at the time of MMO addition. Tube lengths were again measured after 3 hr in MMO. During this final 3 hr, an additional 10% of the pollen grains in the control germinated, but there was no additional germination in the presence of MMO. Even the lowest concentration of MMO, 20 mM, greatly inhibited tube elongation.

MI mitigated the toxic effect of MMO. To demonstrate this effect, germinated pollen was grown at a series of MI concentrations in media containing 30 mM MMO. For comparison, pollen samples were also grown in the absence of MMO. The data are collected in Table II. Lily pollen contains 3 to 5 mg of free MI/g fresh pollen (M-A. Mitchell, unpublished observation). Protection against MMO toxicity provided by this endogenous MI was evident in results from samples grown in media with less than 0.14 mM MI. Above 0.28 mM MI, germination in 30 mM MMO was equal to the untreated control. At 2.78 mM MI, germination was stimulated, despite the presence of MMO. Only partial protection of tube elongation to MMO inhibition was provided by MI and this effect was lost during the final 3 hr of incubation. Whether this loss of protection was due to slow irreversible inhibition of vital growth-promoting processes or to the loss of MI through metabolic interconversions was not established.

The partial protection provided by MI against loss of germinating ability due to MMO is clearly seen in Table III. Here, the protective effect of 0.56 mM MI was compared with controls over a range of MMO concentrations from 30 to 75 mM. MI also partially relieved the inhibition of tube elongation. In this experiment, MMO and MI were present in the media when germination was initiated. In most subsequent experiments involving uptake and incorporation of MI-2-^{3}H and D-glucose-1-^{14}C, pollen germination preceded exposure to MMO and addition of label.

Table IV summarizes results from five experiments in which MI-2-^{3}H was administered to MMO-treated pollen. Each experiment was accompanied by its own untreated control. Samples were run in duplicate or triplicate. In experiments A and B, MMO reduced the total uptake to one-third and incorporation of label into pollen tube polysaccharides to one-fourth of the control. In experiment C where MMO and MI-2-^{3}H were present throughout the growth period, MMO-treated tubes incorporated only $^{1}/_{10}$ of the radioactivity found in the controls and the

Table I. *Effect of MMO on Tube Elongation in Germinated L. longiflorum Pollen*

MMO Concn.	Before Addition of MMO (5 hr incubation)		After 3 hr in MMO-Containing Media
	Germination[1]	Tube Length[1]	Tube Length[1]
mM	%	*mm*	*mm*
0	40	0.50	0.96
20	40	0.55	0.62
30	36	0.39	0.52
35	48	0.52	0.60

[1]Average of 5 samples. Limits were ± 5% for germination and ± 0.02 mm for tube length.

Table II. *Effect of MI on Inhibition by MMO of Tube Elongation of Pollen from L. longiflorum*

MI Concn.	Germination[1] at 5 hr	Tube Length[1]	
		At 5 hr	At 8 hr
mM	%	*mm*	
0 (MMO absent)	57	0.89	1.16
0 (MMO present)	46	0.32	0.32
0.03	46	0.32	0.31
0.14	41	0.29	0.33
0.28	56	0.46	0.48
0.56	56	0.51	0.53
1.67	60	0.72	not determined
2.78	84	0.80	not determined

[1]Statistical limits are the same as Table I.

Table III. *Effect of MMO on Germination and Tube Elongation of Pollen from L. longiflorum Grown in the Presence of MI*

MMO Concn.	Germination[1]		Tube Length[1]	
	MI Absent	MI Present[2]	MI Absent	MI Present[2]
mM	%		*mm*	
0	56	57	0.89	0.95
30	44	55	0.31	0.52
45	29	50	0.18	0.31
60	20	34	0.15	0.24
75	not determined	25		0.19

[1]Statistical limits are the same as in Table I.
[2]MI concentration, 0.56 mM.

Table IV. *Effect of MMO on Uptake and Incorporation of Tritium from MI-2-^{3}H into Germinated Pollen of L. longiflorum*

Experiment	Total Growth Period	MI Concn.	MMO Concn.	Radioactivity Recovered	
				Total	70% Ethyl Alcohol Insoluble Residue
	hr	*mM*	*mM*	*% of tritum supplied*	
A[1]	9	0.006	0	19.3 ± 0.5	10.8
	9	0.006	30	7.1 ± 0.3	2.7
B[1]	9	0.006	0	34.2	13.5
	9	0.006	45	12.0	3.1
C[2]	11	0.056	0	75 ± 0.4	52
	11	0.056	30	7.5 ± 0.6	3.5
D[3]	4	1.11	0	3.9 ± 0.7	0.9
	4	1.11	24	4.4 ± 0.1	0.9
E[3]	5	1.11	0	9.6 ± 2.4	3.0
	5	1.11	24	8.8 ± 1.1	3.0

[1]Pollen was allowed to germinate in basic media for 3 hr. At 3 hr, MMO was added to one set and incubation was resumed. At 6 hr, media in both sets was replaced with fresh media containing MI-2-^{3}H and incubation continued.

[2]Both MMO and MI-2-^{3}H were present at the start of germination.

[3]In Experiment D, pollen was allowed to germinate for 3.5 hr prior to addition of MMO and MI-2-^{3}H. In Experiment E, the same conditions were used with the exception that MMO was added 0.5 hr after MI-2-^{3}H.

proportion entering tube polysaccharides was even less. Raising the MI concentration sufficient to overcome partially the effects of MMO on tube elongation (experiments D and E) restored normal uptake and incorporation at short periods of exposure to MMO. In other experiments not reported here, prolonged incubation of pollen tubes in MMO-containing media led to reduced uptake of MI-2-^{3}H even when the MI concentration was above 1 mM.

The 70% ethyl alcohol-insoluble residues from experiment A were hydrolyzed with pectinase and separated into acidic and neutral constituents on an ion exchange column. A radiochromatographic scan of the neutral sugars is shown in Figure 1. Between 55 and 60% of the tritium appeared in these sugars. The remaining radioactivity, recovered after elution of the anionic column with 3 N formic acid, was galacturonic acid.

To obtain a kinetic view of inhibition of MI-2-^{3}H metabolism by MMO, pollen samples which had been germinated for 3 hr in Dickinson's pentaerythritol media were labeled for 2 hr with MI-2-^{3}H over a range of MI concentrations from 0.1 to 2.8 mM in the presence of 0, 19, and 38 mM MMO. Acid hydrolysis of 70% ethyl alcohol-insoluble residues released all bound radioactivity into solution. Results are shown as a double reciprocal plot in Figure 2. Each point was obtained as the average of triplicate samples. Although the rate-limiting step in conversion of MI to pollen tube polysaccharides in unknown, these data suggest that MMO inhibition of MI metabolism at that step is competitive.

If MMO inhibits conversion of MI to UDP-D-glucuronate and subsequent metabolic products but does not interfere with UDP-D-glucuronate biosynthesis through UDP-D-glucose, use of this inhibitor becomes a valuable means of assessing the functional role of the MI oxidation pathway in germinated lily pollen. To test this possibility, it was necessary to examine the effect of MMO on other key enzymes between D-glucose and UDP-D-glucuronate, notably MI-1-P synthase and UDP-D-glucose dehydrogenase. MMO failed to inhibit partially purified MI-1-P synthase from *A. pseudoplatanus* (M. W. Loewus, unpublished observation) in the concentration range used in the present study. Similarly, the activity of partially purified UDP-D-glucose dehydrogenase from *L. longiflorum* pollen was unaffected by the presence of 16 mM or 32 mM MMO. These observations offer assurance that MMO inhibition of pectin biosynthesis from D-glucose would result from its antagonistic effects on MI oxidation in the MI oxidation pathway and not from interference with

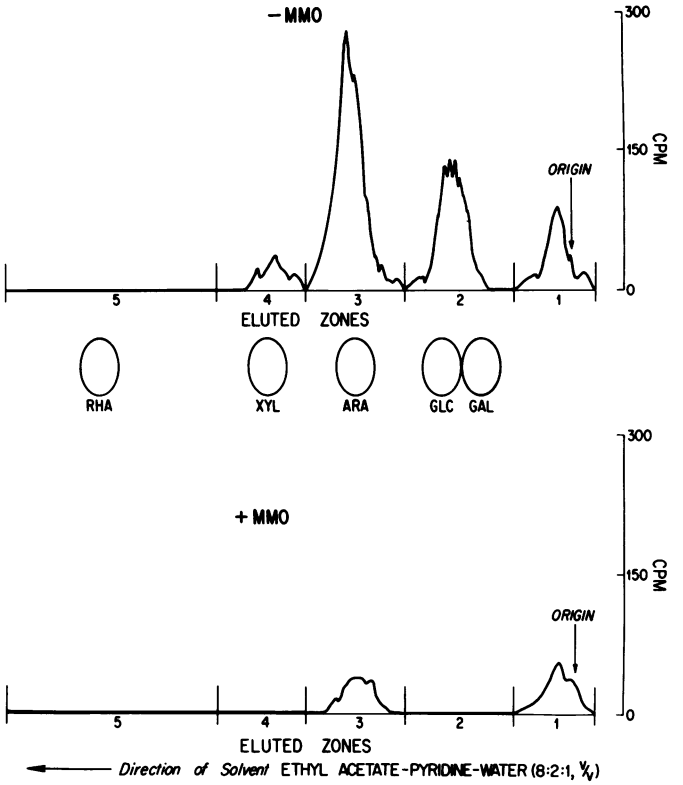


FIG. 1. Radiochromatographic scans of neutral components in the pectinase hydrolysate of MI-3-^{3}H-labeled pollen tubes that were untreated or treated with 30 mM MMO. Locations of standard sugar spots are given in the space between scans.

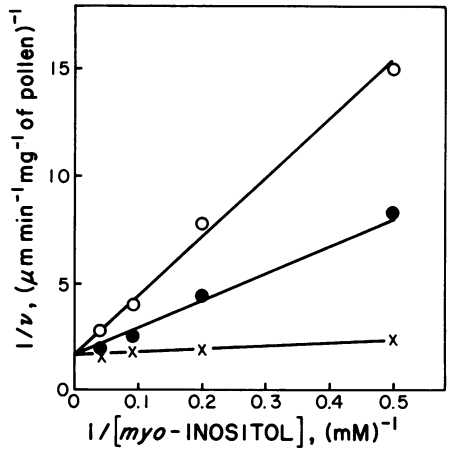


FIG. 2. Double reciprocal plot of incorporation of label from MI-2-^{3}H into pollen tube polysaccharides at X: 0 mM; ●: 19 mM; and O: 38 mM MMO.

the oxidation of UDP-D-glucose in the sugar nucleotide oxidation pathway.

Conditions resembling those used in experiments A and B (Table IV) were used. After 3 hr of germination and 3 hr of exposure to 30 mM MMO, pollen was labeled with D-glucose-1-^{14}C (3 μg, 10^6 cpm) for 3 hr. In experiment F, the MMO-containing medium was replaced with fresh medium at the time of labeling. In experiment G, MMO remained in contact with the pollen throughout the labeling period. About two-thirds of the D-glucose-1-^{14}C was taken up by germinated pollen tubes during the labeling period in experiment F. In experiment G, where pollen tubes were undisturbed by replacement of media, controls reached 81% uptake while MMO-treated samples took

up 67%. In every case, incorporation of label into 70% ethyl alcohol-insoluble residues was 36 ± 3% of the ^{14}C supplied. MMO did not inhibit net incorporation of label.

Enzymic or acid hydrolysis of 70% ethyl alcohol-insoluble residues from experiment F solubilized virtually all radioactivity. Separation of acidic and neutral constituents by ion exchange columns and paper chromatography revealed significant changes in the distribution pattern of ^{14}C after MO treatment (Table V). In experiment F, only 3% of the ^{14}C in the polysaccharides from MMO-treated pollen tubes was retained by the anionic resin as compared to 23% in the untreated sample. All of this acidic material was eluted from the resin with 3 N formic acid as a single peak that had the chromatographic properties of galacturonic acid. In neutral sugar residues, a significant decrease in the incorporation of ^{14}C into arabinosyl units was found in MMO-treated tissues (Fig. 3). Xylosyl and rhamnosyl units also had less label. This ^{14}C which failed to reach galacturonosyl, arabinosyl, and xylosyl units piled up in the glucose-galactose region of the

Table V. *Effect of MMO on Distribution of ^{14}C in Hydrolyzed Products of 70% Ethyl Alcohol Insoluble Residues from D-Glucose-1-^{14}C-Labeled Pollen of L. longiflorum*

Fraction	Experiment F		Experiment G			
	Pectinase		Pectinase		Acid	
	MMO Absent	MMO Present	MMO Absent	MMO Present	MMO Absent	MMO Present
	% of ^{14}C present in hydrolysate					
Acids[1]	23	3	12	4	12	4
Neutrals[2]						
Origin	1	1	1	<1	5	1
Glc/Gal	68	92	75	91	69	89
Ara	6	2	9	3	9	3
Xyl	1	<1	1	<1	1	1
Rha	1	<1	2	1	3	1

[1] Separation was made on Dowex-1-formate (7).
[2] Separation was made by paper chromatography in ethyl acetate-pyridine-water, 8:2:1, v/v. Fractions correspond to regions occupied by sugar standards.

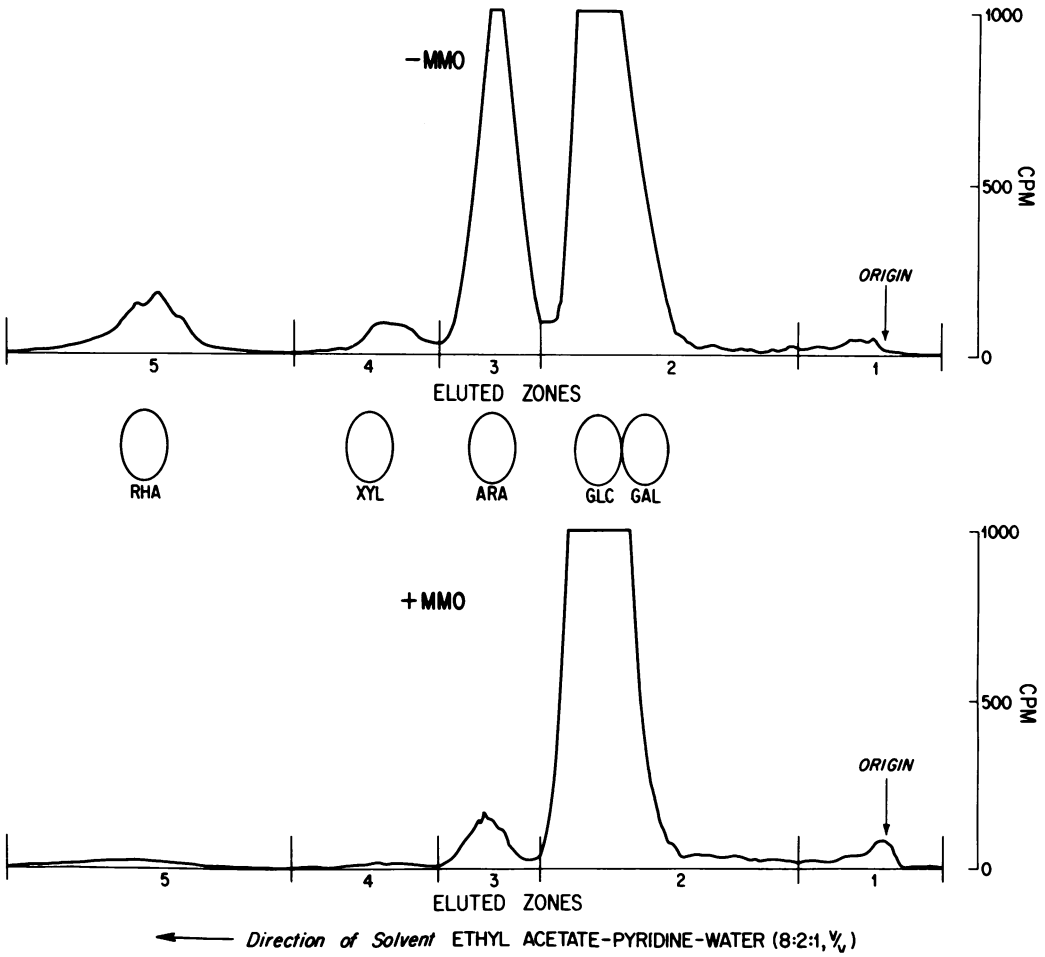
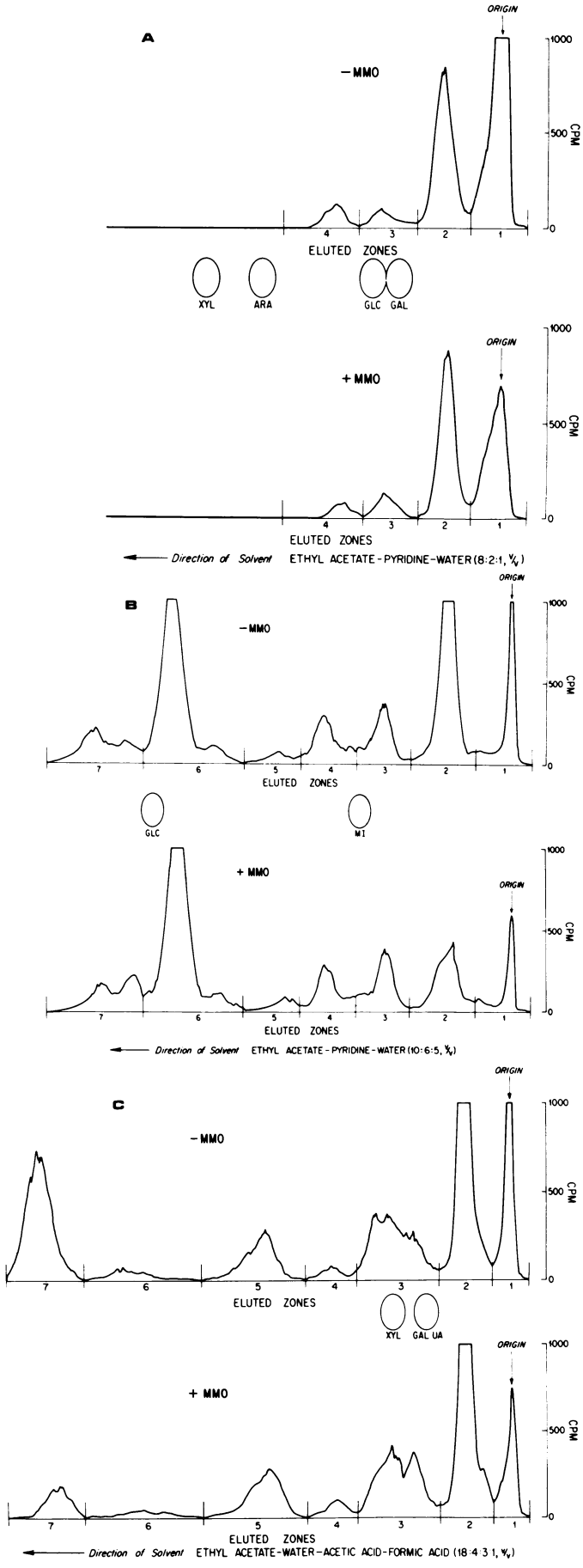


FIG. 3. Radiochromatographic scans of neutral components in the pectinase hydrolysate of D-glucose-1-^{14}C-labeled pollen tubes that were untreated or treated with 30 mM MMO. Locations of standard sugar spots are given in the space between scans.



paper chromatogram, but positive identification of component sugars must still be made in this fraction. Results comparable to experiment F were obtained in experiment G.

The low incorporation of label into galacturonic acid, arabinose, and xylose residues of 70% ethyl alcohol-insoluble residue in glucose-labeled, MMO-treated pollen resembles results obtained with MI-2-^{3}H as seen in Figure 1. Less label appeared in rhamnose, an observation that suggests a process of rhamnosyl-galacturonan biosynthesis tightly controlled by the supply of galacturonic acid precursor since rhamnose biosynthesis is probably unaffected by MMO.

MMO also altered the distribution of at least one labeled acidic component of 70% ethyl alcohol-soluble products from D-glucose-1-^{14}C-labeled pollen. The effect was discovered during paper chromatography of the soluble products. In Figure 4A, less ^{14}C remained at the origin in MMO-treated samples than in untreated samples, while more ^{14}C was found in zone 2 of the treated samples. When the solvent ratio was changed to a more polar mixture to separate hexoses from MI, the affected label previously found on the origin in untreated samples using the less polar system now appeared in zone 2, while that previously within zone 2 in MMO-treated samples using the less polar system now appeared in zone 6 (Fig. 4B). With the aid of a solvent system normally used to separate acidic components, the compound(s) affected by MMO appeared in zone 7 in untreated samples, but after treatment with MMO, more ^{14}C appeared in zone 2 (Fig. 4C).

Paper electrophoresis of labeled material from zone 7 of the untreated sample in Figure 4C in 0.1 M ammonium acetate (pH 5.8) at 25 v/cm for 75 min produced a broad radioactive peak with a mobility exceeding D-glucuronic acid-1-P, UDP-D-glucuronic acid, and D-galacturonic acid.

DISCUSSION

Unlike the seedling with its attendant processes of root and shoot development, the germinating pollen grain has a relatively simple growth pattern and forms pollen tubes with primary wall structure in a very short period of time. Conversion of MI to pectin in the tube wall is easily adapted to laboratory conditions (1). In the presence of MMO, germination is reduced, tube elongation is inhibited, and utilization of MI for polysaccharide biosynthesis is blocked. Low levels of MI prevent the toxic effects of MMO if the MI is present when MMO is added. Once inhibition has occurred, it appears to be an irreversible process. One possible site of inhibition is MI oxygenase although direct evidence for this suggestion is lacking. Weinhold and Anderson (31) have pointed out the potential alkylating function of MMO and have proposed that inactivation of MI oxygenase from rat kidney by MMO may involve alkylation of the enzyme. As others have pointed out in studies on *S. pombe* and the rat (27, 31), protection by MI appears to be a temporary process. Whether, in the case of pollen, this represents slow irreversible interaction between MMO and a vital enzymic component or a metabolic depletion of added MI has not been determined.

Pollen tube residues remaining after extraction of germinated pollen with 70% ethyl alcohol contain starch as well as tube wall polysaccharides. Rosenfield and Loewus (23) found both groups of polysaccharides to be labeled by MI-2-^{3}H. In the tube wall, pectin accounted for most label, primarily as D-galacturonate and L-arabinose. In starch, tritium was incorporated into D-glucosyl units as a result of recycled label from the hexose phosphate pool. Together, these labeled polysaccharides provide a qualitative picture of MI metabolism in lily pollen, espe-

FIG. 4. Radiochromatographic scans of 70% ethyl alcohol-soluble compounds from D-glucose-1-^{14}C-labeled pollen tubes that were untreated or treated with 30 mM MMO. Location of standard sugar spots are given in the space between scans.

cially in the case of labeled D-glucose residues which could only be derived from MI by oxidative cleavage of the inositol ring and metabolic conversion of the product to D-xylose (23). MMO prevented incorporation of label from MI-2-^{3}H into both groups of polysaccharides.

When untreated and MMO-treated pollen tubes were labeled with D-glucose-1-^{14}C, uptake of ^{14}C was similar under both conditions as was incorporation of label into 70% ethyl alcohol-insoluble residues. MMO did not appear to affect D-glucose transport into pollen.

The distribution of ^{14}C from D-glucose-1-^{14}C into monomers of tube wall polysaccharides was greatly altered by MMO. In MMO-treated tubes, very little ^{14}C appeared in uronic acid, arabinose, xylose, or rhamnose units of pectic substance as compared to controls (Table V and Fig. 3). An increase in labeling of hexose residues accounted for the equivalence in total incorporation between MMO-treated and untreated tubes. It is likely that most of the labeled hexose arises from starch and callose, but this remains to be confirmed experimentally. As pointed out earlier, the MMO-promoted decrease in rhamnose label is probably a consequence of reduced pectin synthesis due to inhibition of galacturonate formation, not a direct effect of MMO on hexose phosphate metabolism.

Formation of a labeled, unknown, acidic compound normally found in the 70% ethyl alcohol-soluble fraction of D-glucose-1-^{14}C-labeled pollen was blocked by MMO. An attempt to identify this substance is currently underway.

Although the present study offered no clue regarding the site(s) of MMO inhibition, it is unlikely that transport of D-glucose into the cell was involved. Uptake of D-glucose was not altered in the presence of MMO. Processes involved in uptake of exogenous MI may be affected by MMO but under normal conditions of growth, the MI requirements of the growing pollen tube would be supplied internally from pollen grain reserves or from D-glucose 6-P via MI 1-P synthase. At the MMO concentrations used in this study, MI-1-P synthase was not inhibited. Since MMO-treated pollen produced only one-third the amount of labeled products of UDP-D-glucuronate metabolism found in untreated pollen, at least two-thirds of the carbon flow from hexose to these products was diverted elsewhere, possibly to starch or callose. The sugar nucleotide oxidation pathway failed to provide an alternate route despite the fact that UDP-D-glucose dehydrogenase was not inhibited by MMO.

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